Synthesis and Properties of a Bipolar, Bisphosphatidyl Ethanolamine That Forms Stable 2-Dimensional Self-Assembled Bilayer Systems and Liposomes

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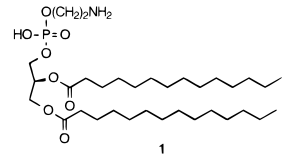
Phosphatidyl ethanolamine is one of the most common naturally occurring phospholipids. The presence of an amino group also makes it one of the most straightforward to functionalize. Unfortunately however, it tends to form micelles and hexagonal clusters instead of forming lamellar systems such as sheets and vesicles (liposomes), key supramolecular structures for a variety of biomedical and other technical applications. This has been overcome by synthesizing a dimeric phosphatidyl ethanolamine molecule in which the acyl chains at the 2-position of glycerol are joined at their termini by a carbon−carbon bond, thus resulting in a trans-membrane type fatty acyl linkage. Such tail-to-tail bipolar transmembrane lipids are found in the membranes of thermophilic and other extremophilic bacteria. The dimeric phosphatidyl ethanolamine readily forms very uniformly flat self-assembled lamellar supramolecular arrays and liposomes that are stable at temperatures up to 80 °C. The planar systems formed by the new lipid were characterized by atomic force microscopy, and the uniformity of the vesicles was observed by laser scanning confocal microscopy. The thermal stability of the phospholipid and the vesicles formed was examined by NMR spectroscopy including proton T_1 measurements as a function of temperature. Fourier transform infrared spectroscopy indicated a much greater order of the alkyl chain in the dimer than in phosphatidyl ethanolamine. This extremely stable and readily functionalizable dimeric phospholipid has potential uses in the fabrication of biomaterials, stable membrane models, and liposome drug-delivery systems.

Introduction

There has recently been much interest in the use of phospholipids, phospholipid analogues, and other systems that form 2-dimensional lamellar systems in several areas of research and technology including drug encapsulation,[1,2] gene delivery,[3,4] biocompatibilty,[5,6] sensors and specialized receptor surfaces,[7,8] and advanced biomaterials.[9−14] Phospholipids serve as the prototypical materials for the design of such surfaces because of their extremely high packing densities and propensity to form lamellar systems. In all of these applications, it is desirable to form a self-assembled lamellar system with some desired functionality on one surface. In the drug encapsulation and gene delivery areas, it is a requirement that the lamellar system have an overall spherical topography to form hollow structures called vesicles or liposomes. From the standpoint of chemical functionality, of all of the naturally occurring phospholipid systems, none is more suited for such applications than phosphatidyl ethanolamine **1**. This is because of the presence of



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the easily derivatizable primary amino group. Unfortunately, because of the small size of its headgroup, phosphatidyl ethanolamine tends not to form lamellar systems but forms micelles or hexagonal phases instead.[15] It is therefore not possible to make preformed lamellar systems and then functionalize them.

One general drawback to the use of phospholipids in the applications discussed above is the general instability of the supramolecular structures they form. Hence, vesicles tend to lyse too easily and leak the materials that are trapped therein. Planar lamellar systems on solid supports often lack sufficient intermolecular cohesive power and are too easily perturbed. Some special device such as a Langmuir−Blodgett trough is often required to form them in the first place. It is therefore important

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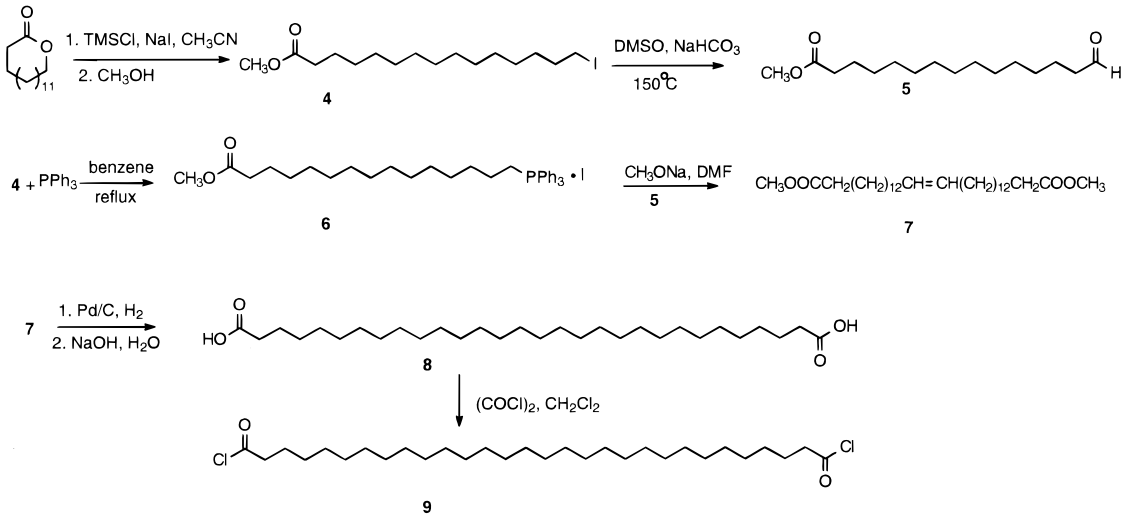
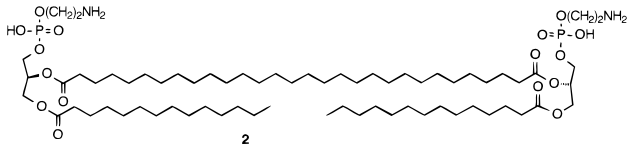
Scheme 1. Synthesis of Triacotanedioic Acid 8



to design and synthesize phospholipid variant structures that contain some extra stabilizing features. The preparation and use of such materials is of special significance to several very different fields.

There are good clues as to how to stabilize the lamellar bilayer structure of phospholipids if one examines the structures of biomembranes of organisms from habitats where extremes of temperature or pH or the presence of deleterious chemical substances persist. Such organisms can flourish at temperatures in excess of 100 °C in geothermally active sites at the bottom of the ocean. For such organisms, one common structural feature of their membrane lipids is the presence of transmembrane fatty acyl components.[16−18] These lipids contain very long (28 carbons or more) α-*ω*-dicarboxylic acids formed by tail-to-tail joining of lipid fatty acyl chains between the two leaflets of the bilayer.[18−19] The synthesis of lipids such as **2** with the phosphoethanolamine headgroups is an



excellent target. Such molecules are not known but would combine the good thermal and chemical stability associated with the presence of the very long α-*ω*-dicarboxylic acid group with the ease of modification of the primary amine group. In addition, any tendency to form non-lamellar systems such as micelles would be suppressed. Because of the stabilization by ionic forces at both ends and the extra stabilization by the very long hydrocarbon chains, it should not be necessary to compress films of these lipids molecules into compact layers using Langmuir−Blodgett troughs. This approach has been used with synthetic bolaform amphiphile models of archaebacteria membrane lipids,[20−24] which also contain transmembrane alkyl chains, except that they are ether linked to the headgroups. These tetra ether bipolar lipids have the advantage of forming lamellar systems that are stable to extreme pH,[25] high temperatures,[21,26] and high ionic strengths.[27] However, these models have ether instead of ester linkages connecting the hydrocarbon chains to the headgroup and lack many of the structural features of biologically important lipids. This might be critical for medical applications. We describe herein the preparation and characterization of the transmembrane stabilized phosphatidyl ethanolamine **2** containing triacotanedioic acid. The lipid contains normal phosphatidyl ethanolamine headgroups and ester linkages. Because archaebacteria lipids contain ether linkages between the hydrocarbon chains and the headgroups, release of encapsulated substances by the action of phospholipase A-type activities (esterases) is not possible. This may limit the practicality of using such liposome systems in applications where host enzymes mediate the release of the trapped substance.

Results and Discussion

Synthesis of the Transmembrane Phospholipid Analogue 2. The synthesis of **2** involved first the preparation of triacotanedioic acid followed by its coupling to a protected *lyso*-lipid. Triacotanedioic acid was prepared from pentadecalactone according to Scheme 1. The lactone was converted to the 15-iodoacid **4**, and a portion of the acid oxidized to the aldehyde **5**. The aldehyde and iodoacid (protected as esters) were joined by Wittig reaction to form triacotan-15-ene dioic acid

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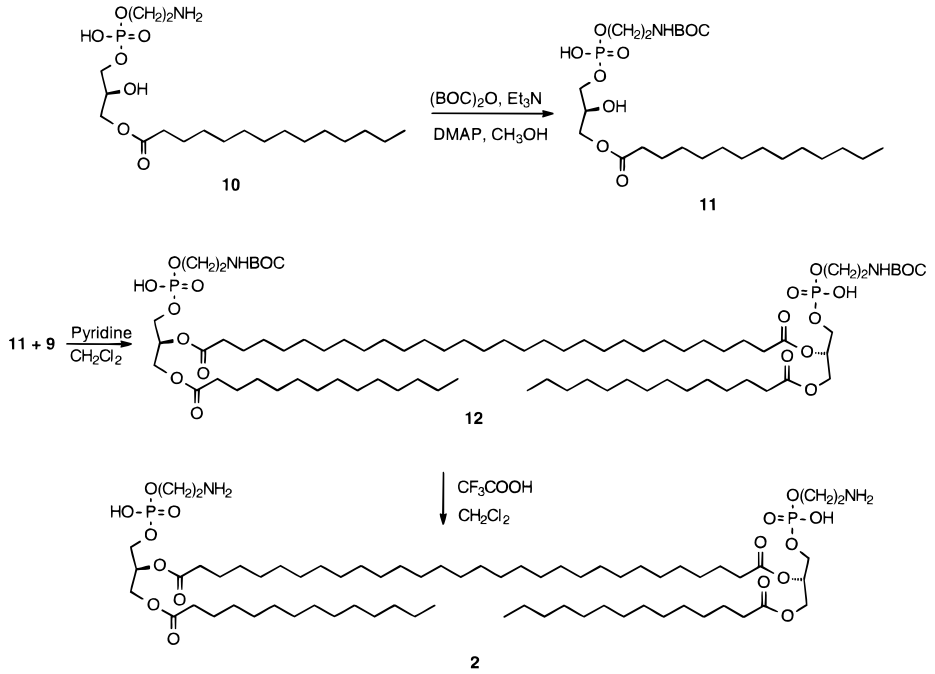
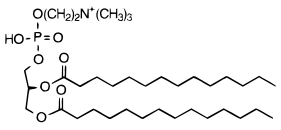
Scheme 2. Synthesis of Dimeric Phosphatidyl Ethanolamine 2



dimethyl ester, which was converted to triacotanedioic acid dimethyl ester by catalytic hydrogenation. Saponification and acidification yielded the diacid, which was converted to the acyl chloride **9** with oxalyl chloride. The transmembrane stabilized phospholipid **2** was then constructed as shown in Scheme 2. Two molecules (**11)** of amino-protected (*tert*-butyloxycarbonyl) *lyso*-phosphatidyl ethanolamine with a tetradecanoyl group in the 1-position were joined by one molecule of the acid chloride of triacotanedioic acid. The desired compound **2** was obtained after removal of the *tert*-butyloxycarbonyl group with trifluoroacetic acid.

Supramolecular Structure, Chain Packing, and Film-Forming Properties. The fully hydrated films formed by compound **2** and a typical phospholipid that forms lamellar systems and vesicles easily (phosphatidyl choline **3**) were studied by laser confocal scanning



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microscopy. The images were observed using polarized light, dark field, and phase contrast optics. The results are shown in Figure 1. The lipid **2** exhibited very different behavior compared to that of the normal phospholipid **3**. Whereas the latter formed sheets, ribbons, and vesicles with a large variation in size, lipid **2** formed only vesicles with a very narrow distribution in size.

The atomic force micrographs (Figure 2) of thin films formed by compound **2** on mica plates demonstrated that they were extremely flat and uniform with a maximum surface variation of only 5.8 nm over a distance of 800 nm (0.7%). This ability to form 2-dimensional systems with such low surface variation has great significance for the potential use of lipid **2** in a variety of applications. The presence of the free primary amino headgroup should allow surfaces modified with this lipid to be further functionalized with a variety of reagents, especially peptides and proteins with specific biological functions. The amino groups can also be functionalized with various optical probes and a myriad of other structures. Such surfaces have potential value in nanofabrication, surface patterning, advanced material science, and the construction of biological membrane mimics.

Information about intermolecular chain packing and intramolecular chain conformation of phospholipids and other membrane-forming amphiphiles can be obtained by vibrational spectroscopy methods such as Raman and infrared spectroscopy.[28−31] It is known that for hydrocarbon chains the intensity ratio of the methylene asymmetric (2918 cm^{-1}) vibrations over the symmetric (2850 cm^{-1}) is sensitive to the intermolecular chain packing order and the ratio increases with increasing order.[31] Fourier transform infrared spectroscopy experiments were carried out on lipid **2** and on dimyristoyl phosphatidyl ethanolamine **1** in both the dry and the hydrated states. The peaks were deconvoluted and quantified by Fourier analysis in conjunction with a curve-fitting routine using a mixed Laurentian and Gaussian line shape and a peak width parameter that could be adjusted to give optimum fitting. Analysis on the dry films of dimyristoyl phosphatidyl ethanolamine gave an area ratio (asymmetric to symmetric) of 1.83, whereas the value obtained for the dimer was 2.58. The hydrated samples (prepared by forming the films from water by slow evaporation onto a calcium fluoride window) gave a ratio of 1.37 for dimyristoyl phosphatidyl ethanolamine and 2.33 for compound **2**. These results demonstrated a substantially higher ordering of the alkyl chains in the dimeric phospholipid compared to dimyristoyl phosphatidyl ethanolamine in both dry and hydrated states. In the

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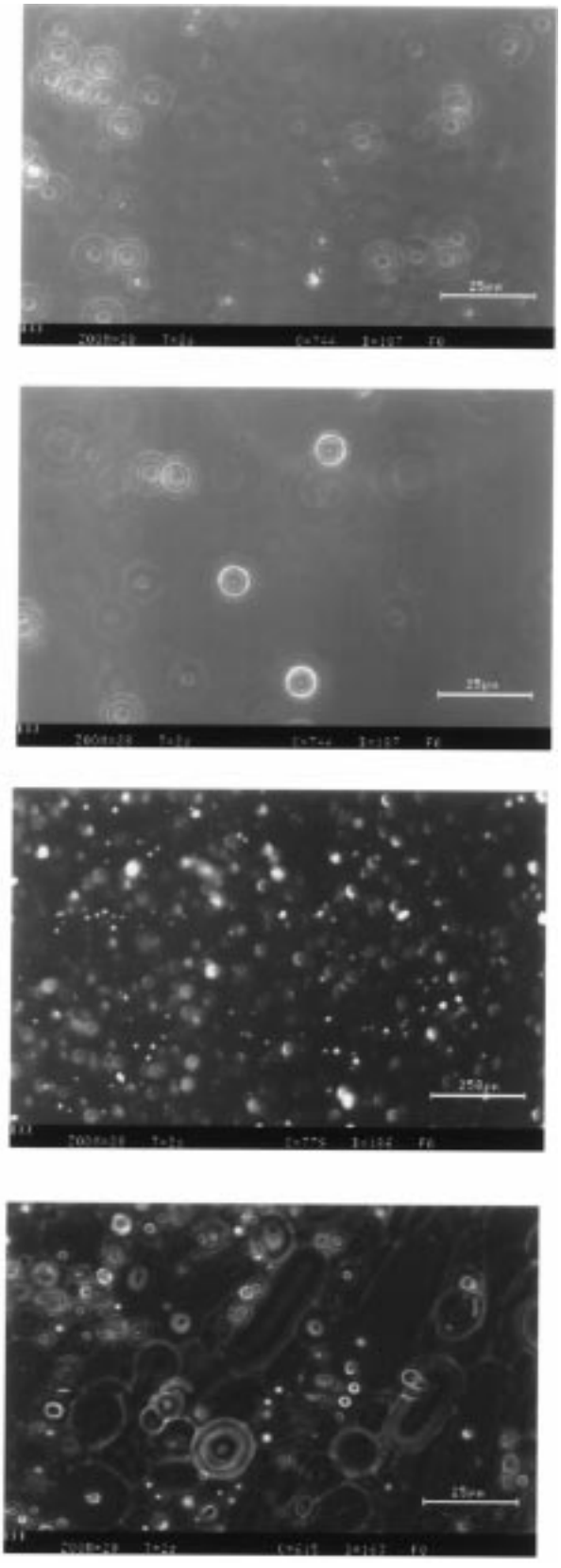

Figure 1. Laser scanning confocal microscopy images of hydrated compounds **2** and **3**. The first and second images are phase contrast pictures of **2**. The circular structures are liposomes. Note the uniformity of their size. The third image is a dark field image of compound **2** showing the spherical outline of the liposomes. The last (bottom) image is a phase contrast picture of **3**. Note the variation in size of the liposomes, as well as the presence of several other lamellar structures corresponding to sheets and ribbons.

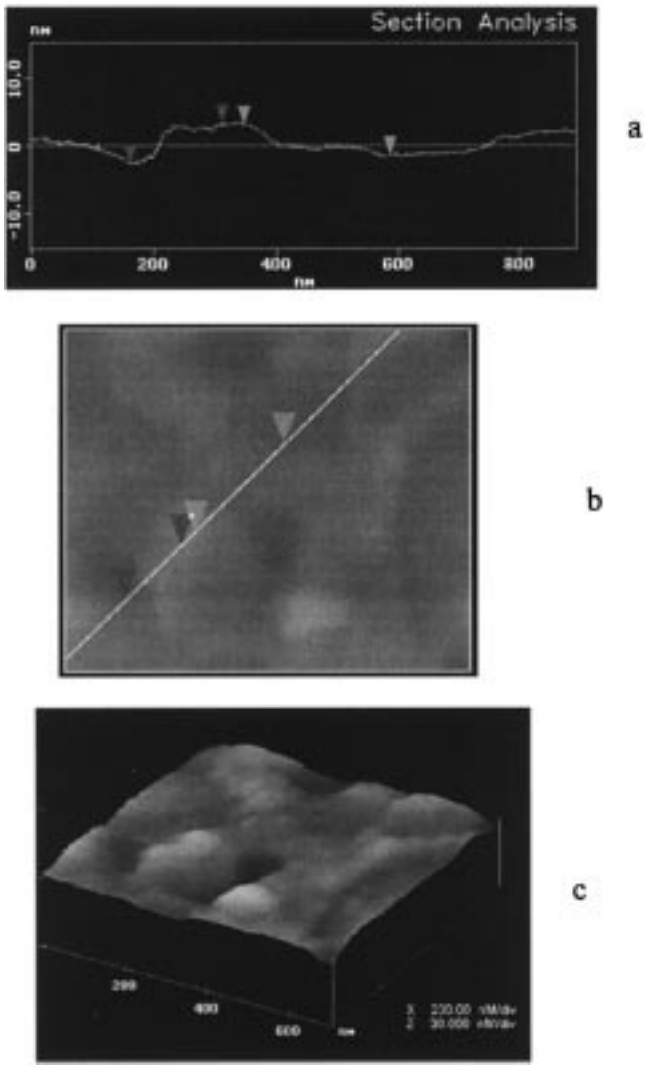

Figure 2. Atomic force micrographs of the thin film formed by compound **2**: (a) section analysis over an 800 nm range, (b) top view of the same area from which the section analalysis was taken, and (c) surface plot of the same area as the above two images.

hydrated state, both lipids showed a small decrease in order, but the dimer was still significantly better packed than the normal phospholipid.

Self-Assembly Properties, Stability, and Liposome Formation. The structural order and stability of the supramolecular systems formed by lipid **2** were probed by two different nuclear magnetic resonance (NMR) spectroscopy experiments. NMR spectroscopy is a very useful tool for studying biomembrane systems.[16,17,20] The motional freedom of specific groups in the lipid can be determined by measuring the parameter referred to as T_1 or the spin lattice relaxation time. It is also known as the longitudinal relaxation time. This parameter describes the rate of magnetization transfer from a nucleus at its high energy state to its environment via a dipolar coupling mechanism. If the system is rigid and/or ordered, this transfer is effective and T_1 is short. When the system is less rigid or structured, the nucleus may not be close to any other one for long enough (on average) to facilitate good transfer and the value of T_1 is then larger. If T_1 is evaluated as a function of temperature, a sudden, simultaneous change in its value at any

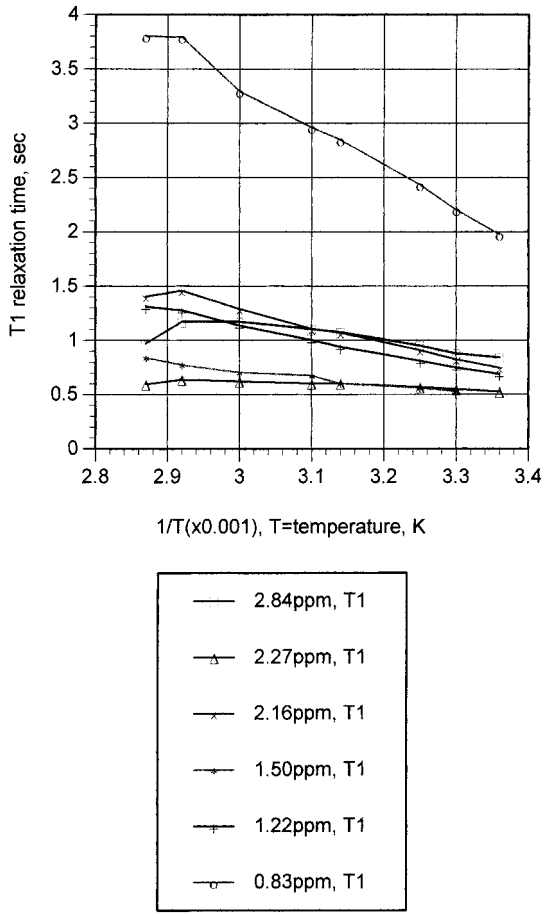

Figure 3. The relationship of T_1 relaxation time versus the reciprocal of temperature ($1/T$) for bulk methylene (1.22 ppm), methyl (0.83 ppm), methylene next to amino groups (2.84 ppm), methylene α to carbonyl groups (2.27 and 2.16 ppm), and methylene β to carbonyl groups (1.55 ppm).

given temperature for more than one signal would indicate a change in phase of the system. T_1 relaxation times of **2** in deuterated DMSO at various temperatures were measured. The curves for the dependence of T_1 on temperatures (actually $1/T$) are shown in Figure 3. As expected, the terminal methyl groups displayed the largest T_1 values among all the groups present in the lipid. In general, the value of T_1 increased with temperature. There was a discontinuity in the plot of T_1 vs temperature for the methyl signals at 70 °C, indicating a phase transition. No discontinuity was observed for the curve corresponding to the bulk methylene groups up to a temperature of 75 °C. Two signals corresponding to the methylene protons adjacent to the ester carbonyl groups were observed at 2.16 and 2.27 ppm. The nuclei giving rise to the first signal had a significantly larger T_1 value, indicating that it was most likely due to the protons on the more mobile 14-carbon chain. A discontinuity was observed for both signals at 70 °C. In the headgroup region, the signals for the methylene group adjacent to the amino group displayed a larger T_1 value, indicating that it was more mobile than the other methylene group. A discontinuity at 70 °C was also observed. The concerted abrupt change in T_1 at 70 °C is indicative of a significant change in phase at this temperature. It indicates that there is a high degree of supramolecular organization in lipid **2** even in dissociating solvents and at temperatures way above those at which normal lipids function.

Liposomes or vesicles have been explored for drug delivery models because of their ability to encapsulate various components inside. Because of their instability, liposomes made from typical lipids are not good drug carriers, and many methods have been developed to improve the stability of liposomes.[32,33] Lipids isolated from thermophilic bacteria were used to prepare liposomes which proved to have increased resistance to leakage.[34−36] To explore the stability of a liposome formed from the stabilized phospholipid dimer **2** as a drug delivery system, the ability to trap water (H_2O) and ferric ions (paramagnetic relaxing agents) inside such a liposome was studied by proton NMR spectroscopy. The spectra of a suspension of these liposomes were obtained in D_2O with increasing temperature with the expectation that when the temperature was high enough to cause leakage, the ferric ions would leak out and cause a sudden increase in line width of the bulk external water line. The liposomes were prepared according to literature methods[37−40] with modifications as described in the Experimental Section. There was one striking feature of the NMR spectra (Figure 4). The signal for the water line at 20 °C was broad and triangular in shape indicating that the water was restricted in motion and confined to a region with significant chemical shift anisotropy. This was very reminiscent of the peak shape for phosphorus signals in phospholipids,[41,42] in which the shape stems from the same cause. Closer inspection of the peak revealed that there were several envelopes superimposed on each other. These signals were due to trapped water in different environments inside of the vesicles. As the temperature was increased, the envelopes became more discreet as the individual peaks narrowed because of increased mobility of the water molecules. As the temperature was increased, there was also a gradual exchange of H_2O from inside for D_2O from outside of the vesicles. This was indicated by a gradual disappearance of some of the signals. The presence of multiple water peaks even at 80 °C indicated how resistant to breakage and leakage the liposomes were even at that temperature. Breakage would have led to an immediate reduction of all of the signals to one broad signal. It was clear from the experiments that the ferric ions were still trapped inside of the vesicles even at this temperature.

Conclusion

In these studies, we have synthesized a new membrane-spanning dimeric phosphatidyl ethanolamine molecule

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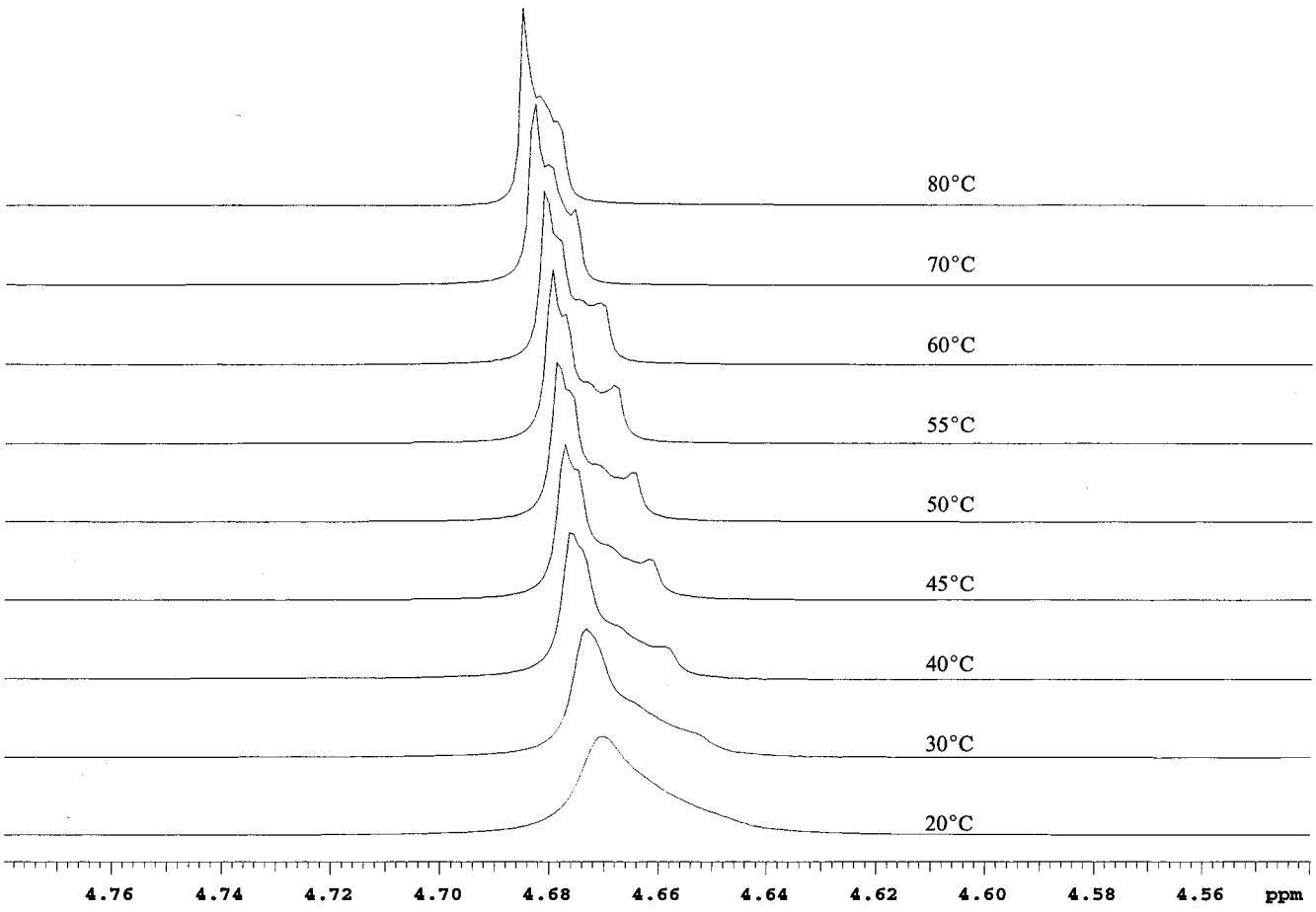

Figure 4. 300 MHz ^{1}H NMR spectra of the water signals in a liposome suspension of compound **2** in PBS−deuterium oxide at different temperatures.

that readily forms stable vesicles of uniform size, as well as a flat, thin lamellar film with very small variation in surface features. The motion of the lipids in these systems is considerably more restricted and the alkyl chains are more ordered than those in typical phospholipid systems in both the dry and hydrated states. The lipid has good thermal stability and high phase transition temperature. The vesicles fabricated from this tail-to-tail dimer lipid are extremely uniform in size and very stable to high temperatures. The results we obtained here are important because phosphatidyl ethanolamine does not form vesicles on its own. The synthesis of phospholipids in general is difficult, and the synthesis of ether-containing archaebacteria-type lipids is even more so. Lipids from archaebacteria are not generally available in significant quantities. Naturally occurring phospholipids are available in substantial quantities, and the strategy we adopt here can be employed if the *sn*-2 fatty acyl groups are removed with a phospholipase A enzyme. The approach we describe here is generally applicable to other *lyso*-phospholipids provided that the headgroups can be protected. These ultrastable membranes can be further developed for fabricating stabilized drug-delivery systems. They also have potential for the preparation of biocompatible surfaces and the fabrication of molecular devices through modification of the primary amino group, perhaps with metal-binding functional groups. There are also possibilities for other surface-chemistry applications in material science and biotechnology.

Experimental Section

Atomic Force Microscopy. These analyses were performed using a Nanoscope III instrument operating in contact mode. For these measurements, compound **2** was dissolved in chloroform (0.5−1% solutions), and ~10 μL was used to coat freshly cleaved mica plates spinning at 200 rpm to effect uniform coating.

Laser Scanning Confocal Light Microscopy. These experiments were performed on a Zeiss 210 instrument with a 488 nm laser. Images were obtained in the dark-field, phase contrast and polarization modes. For the polarizing mode experiments, an analyzing cross-polarizer was placed on the objective lens and rotated until light cancellation. For film preparation, the compound was dissolved in chloroform and deposited on a microslide. After the solvent evaporated, a drop of water was added on top of the film, and the film was hydrated for 2−3 days. Before the measurements, a drop of water was added on top of the film, which was then covered with a round cover glass. For comparison, a sample using PC (compound **3**) was prepared and observed by the same methods.

Vesicle Stability Experiment. The vesicles were prepared according to methods in the literature[37−40] with modifications. About 1−2 mg of compound **2** was suspended in 0.4 mL of phosphate buffer solution (PBS) (Dulbecco, pH = 7) in a small vial, and approximately 50 μL of 0.1 M $MgSO_4$ solution and 3−4 mg of ferric citrate were added to the vial. The mixture was then sonicated and vortexed alternatingly for about 45 min. It was then allowed to stand at room temperature for 20 h before being dialyzed against PBS. Just before the NMR spectroscopy measurements, the water was exchanged with PBS made up in deuterated water. Approximately 50 μL of

this liposome suspension was added to 0.6 mL of PBS in deuterated oxide for the NMR study.

FTIR Experiment. The FTIR experiments were performed on Nicolet 710 spectrometer. For the dry state measurements, the sample was dissolved in chloroform and the solution was transferred to a sodium chloride window, where the solvent was allowed to evaporate. For measurements in the hydrated state, the sample was dissolved in water, which was sonicated to form a suspension, a sample of which was placed on a calcium fluoride window. The suspension was air-dried for 3−4 h to remove the excess water. Heating and reduced pressure is generally required to remove the hydration water. The deconvolution of the methylene symmetrical and asymmetrical absorptions and the calculation of peak area were performed using Galacitic Peaksolve program by Galactic Industries Corporation.

T_1 Relaxation Experiments. Proton T_1 measurements were performed on a Varian 300 MHz NMR spectrometer. Measurements were made at temperatures of 25, 30, 35, 40, 45, 50, 60, 70, and 75 °C. The parameters for each temperature were the same. Fourteen points were collected for each relaxation curve at each temperature. The pulse sequence used was π-τ-π/2, pw 90 = 12.7 ms. The solvent used was deuterated DMSO.

Synthesis

15-Iodopentadecanoic Acid Methyl Ester 4. To a 1000 mL round-bottom flask was added pentadecalactone (22.0 g, 0.915 mol), trimethyl silyl chloride (23.2 mL, 0.183 mol), sodium iodide (41.2 g, 0.275 mol), and 220 mL of acetonitrile. The mixture was left stirring at 50 °C for 14 h. Then, 220 mL of methanol was added to the reaction mixture, the heat was turned off, and the mixture was left for another 3 h. The dark brown mixture was filtered, and the liquid was concentrated by rotatory evaporation to remove all solvents. The residue was dissolved in chloroform and washed with 50 mL of saturated sodium thiosulfate, and the organic layer was dried with sodium sulfate. Upon removal of solvent, a white to light yellow solid was obtained 34.6 g (99%); mp 44−45 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.64 (s, 3H), 3.17 (t, 2H, J = 6.9−7.2 Hz), 2.28 (t, 2H, J = 7.5−8.4 Hz), 1.80 (q, 2H, J = 7.2 Hz), 1.59 (q, 2H, J = 7.2 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz), 174.29, 51.40, 34.09, 33.55, 30.49, 29.56, 29.55, 29.51, 29.41, 29.39, 29.23, 29.13, 28.52, 25.72, 24.93, 7.27; IR (NaCl, $CHCl_3$ wavenumber, cm^{-1}), 2916.7, 2851.2, 1734.2, 1473.8, 1457.2, 1250.0, 1202.2, 1169.0, 756.2.

15-Oxopentadecanoic Acid Methyl Ester 5. A mixture of 4 g of sodium hydrogen carbonate and 60 mL of dry dimethyl sulfide was heated to 150 °C under dry nitrogen. To this mixture was added 5.32 g (13.9 mmol) of compound **4**, and heating and stirring continued for 4 min. The flask was cooled quickly, and the mixture was poured into water which was stirred for several hours. The white solid that formed (the aldehyde) was filtered out or recovered by extracting 4 times with ether. The yield of aldehyde **5** was 3.70 g (98.6%). The aldehyde can be purified by chromotography (solvent hexane/acetate = 7:1). The yield after purification is 3.20 g (86.5%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 9.74 (t, 1H, J = 2.0 Hz), 2.40 (d′t, 2H, J = 7.2 Hz, 2.0 Hz), 2.28 (t, 2H, J = 7.5 Hz); ^{13}C NMR ($CDCl_3$, 75 MHZ) δ 202.84, 174.28, 51.36, 43.86, 34.06, 29.52, 29.37, 29.29, 29.19, 29.09, 24.90, 22.04.

Synthesis of the Triacontanedioic Acid 8. Preparation of the Triphenyl Phosphine Salt 6. The methyl ester iodide **4** (7.64 g, 0.02 mol) and 10.48 g (0.004 mol) of triphenyl phosphine were stirred in benzene under refluxing conditions for 24 h, after which the solvent was removed by vacuum distillation. The residue was taken up in diethyl ether, and the solution was stirred vigorously for at least 1 h. The mixture was filtered to remove the triphenyl phosphine in ether, and the white solid was collected and washed with ether again to ensure that all the excess triphenyl phosphine was removed. The product **6** was dried in a vacuum oven for 24 h. The yield was 11.6 g (90%). The salt **6** (6.4 g) and 20 mL of anhydrous dimethylsformamide were mixed in a round-bottom flask. Sodium methoxide (0.6 g) was added very quickly to the solution, which was stirred and cooled in an ice bath for 10 min. After this time, 0.45 g of freshly prepared aldehyde **5** dissolved in 10 mL of dry DMF was added to the mixture in a dropwise fashion under dry nitrogen. The temperature was maintained at 0−5 °C during the addition. The reaction mixture was stirred for 24 h, after which time TLC analysis indicated that the reaction was essentially completed. The reaction mixture was diluted with water and extracted with hexane several times. The crude product in the hexane extract was purified by flash column chromatography (hexane/ethyl acetate = 9:1). The product was a white solid, yield 0.74 g (87%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 5.40−5.28 (m, 2H), 3.66 (s, 3H), 2.30 (t, 4H, J = 7.5 Hz), 2.07−1.91 (m, 4H), 1.68−1.54 (m, 4H), 1.25 (s, broad, 40H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 174.32, 129.85, 51.41, 34.08, 29.75, 29.63, 29.56, 29.43, 29.29, 29.24, 29.12, 27.17, 24.93.

The ester was hydrogenated (50−200 psi) using Pd−C (10%) in ethanol to give the saturated compound, which upon saponification and acidification of the sodium salt gave the diacid **8** (0.56 g) as a white solid. The overall yield was 80% for these two steps. ^{1}H NMR (DMSO, 300 MHz) δ 2.149 (t, 4H, J = 7.2 Hz), 1.52−1.36 (m, 4H), 1.32−1.12 (s, broad, 48H); ^{13}C NMR (DMSO, 75 MHz) δ 175.65, 34.50, 29.70, 29.52 (m), 29.32, 25.30; IR ($CHCl_3$, NaCl) 3046(broad), 2915, 2848, 1694, 1471, 1462, 1434, 1408, 1284, 1109, 897.6, 718.5; MS calc for $C_{30}H_{58}O_4$ 482, found ES(+) 505 ($M + Na^+$).

Preparation of Compound 2. Boc Protection of Lipid 9. A 0.55 g (1.29 mmol) portion of phospholipid 1-myristoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine **11** was dissolved in 100 mL of a 1:1 chloroform and methanol mixture. $(Boc)_2O$ (0.6 g), 0.02 g of dimethylamino pyridine, and 0.3 mL of triethylamine were added to the reaction mixture, which was stirred overnight. The solvent was removed, and the colorless liquid was washed with 0.1 N HCl once, saturated sodium bicarbonate solution once, and then by brine. The organic layer was dried overnight by sodium sulfate. After removal of the solvent and drying in a vacuum oven for 24 h, the yield of a light yellow semisolid was 0.65 g (95%). ^{1}H NMR (CD_3OD, 300 MHz) δ 4.07 (dd, 1H, J = 4.5 Hz, 11.7 Hz), 3.99 (dd, 1H, J = 5.7 Hz, 11.7 Hz), 3.85 (m, 1H), 3.01−3.89, 3.72−3.80 (m, 4H), 3.16 (t, 2H, J = 5.4 Hz), 2.24 (t, 2H, J = 7.5 Hz), 1.44−1.57 (m, 2H), 1.32 ppm (s, 9H), 1.18 (s, broad, 20H), 0.79 (t, 3H, 6.6 Hz).

Dicarboxylate acid **8** (0.12 g, 0.25 mmol) in 10 mL of oxalyl chloride and 5 mL of dichloromethane was stirred for 12 h. The solvent was removed quickly, and the resulting acid chloride **9** was dissolved in 5 mL of dry dichloromethane and added to a round-bottom flask that contained phospholipid **11** (0.32 g, 0.61 mmol), 5 mL of dry pyridine, and 10 mL of dichloromethane. The mixture was stirred for 24 h, protected from moisture by a drying tube. The solvent was removed, and the crude product was taken up in chloroform and washed with sodium bicarbonate solution. The organic layer was dried, and the solvent was removed. The crude product was purified by flash column chromatography (chloroform/methanol = 7:3). The purified compound **12** was obtained as a white solid (0.24 g, 65%): ^{1}H NMR (CD_3OD, $CHCl_3$ = 5:1) δ, 5.22 (s, 2H), 4.25−4.10 (m, 4H), 4.10−3.82 (m, 8H), 3.40−3.25 (4H, overlap with D-methanol absorption), 2.35 (t, 8H, J = 7.5 Hz), 1.61 (m, 8H), 1.44 (s, 9H), 1.29 (s, 88H), 0.90 (t, 6.8 Hz); ^{13}C NMR ($CDCl_3$, DEPT) δ 68.8, 66.9, 64.5, 41.0, 34.0, 31.9, 29.7, 29.5, 29.4, 29.2, 28.9, 28.4, 24.8, 22.7, 14.1; IR ($CHCl_3$, NaCl) 3380, 2929, 2856, 1743, 1670, 1524, 1464, 1365, 1246, 1173, 1106, 1067, 954.1.

A sample of **12** (∼50 mg) was deprotected by treatment of a solution in 3 mL of dichloromethane with 3 mL of trifluoroacetic acid. This solution was stirred at room temperature for 4 h, and the solvent and other volatile material formed during the reaction was removed on the rotatory evaporator. Compound **2** was obtained as an off-white solid. The removal of the protecting group was completed as judged by NMR spectroscopy. ^{1}H NMR (CD_3OD, $CHCl_3$ = 5:1) δ 5.24 (m, 2H), 4.30−3.68 (m, 12H), 2.40−2.24 (m, 8H), 1.61 (m, 8H), 1.27 (s, 88H), 0.88 (t, 6H, 6.6 Hz); ^{13}C NMR ($CDCl_3$, DEPT) δ 33.9, 31.9, 29.7, 29.3, 29.2, 24.8, 22.6, 14.0; IR ($CHCl_3$, NaCl) 3397,

3240, 2919, 2851, 1738, 1682, 1468, 1207, 1140, 1078, 1024, 800.0, 771.8, 723.4. MS: calc. for $C_{68}H_{134}N_2P_2O_{16}$ 1297, found ES(−) 1296 (M − 1).

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